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Biosorption of mercury by *Bacillus thuringiensis* (CASKS3) isolated from mangrove sediments of southeast coast India

Kailasam Saranya[1], Arumugam Sundaramanickam[1*], Sudhanshu Shekhar[1] & Sankaran Swaminathan[2]

[1]CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai– 608 502, India

[2]2AVC College of Arts and Science, Mannampandal, Mayiladuthurai - 609 305, India

[*][E.Mail: fish_lar@yahoo.com]

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Present study highlights the fact that the bacterium *B. thuringiensis* CASKS3, isolated from mangrove sediments is an efficient strain for biosorption of mercury. Metal-tolerant bacterial strain CASKS3 was identified as *Bacillus thuringiensis* following 16S rRNA gene sequence analysis. Initially, the strain was tested at a minimal inhibitory concentration (MIC) of mercury and its antibiotic resistance was observed. Bacterium was exposed to different concentrations of mercury ranging from 200 to 800 $mg^{-1.}$ Adsorption ability was observed to be 38–62 at 800 mg^{-1} to 200ppm.

[**Keywords:**Biosorption, Mercury, Mangrove sediment, *Bacillus thuringiensis*]

Introduction

Mercury is one of the most toxic heavy metal, when released into the environment[1] causes a serious threat to humans, animals, and plants[2]. It is recognized as a severe environmental pollutant due to its toxicity and its ability to enter the biological system at negligible concentrations[3]. Aquatic ecosystems and wetlands are susceptible to mercury pollution[4]. In addition to its occurrence in the nature, anthropogenic activities also leads substantial quantities of mercury into the marine environment. Industrial effluents produced by mercury mining, gold smelting, fuel combustion, instrument manufacturing, chloride production, antiseptics, fungicides, and bactericidal agents release mercury into the environment[5].

Several techniques such as chemical precipitation, conventional coagulation, reverse osmosis, ultra-filtration, magnetic filtration, ion exchange, activated carbon adsorption, and chemical reduction are available for the removal of toxic heavy metals from wastewater[6]. Nevertheless, these techniques have some negative impacts on the environment and are also highly expensive.

Bioremediation can be used as an alternative tool for the removal of mercury because it is a simple and cost-effective technique[7]. Among the various biosorption methods, the use of micro-organismsplaysa significant role in the adsorption of heavy metals from contaminated wastewater[8]. Karaca et al. (2010)[9] stated that the use of micro-organisms is an efficient and simple method for removing heavy metals. Hazardous materials can be easily broken down or transformed into simple and non-hazardous compounds. Out of all the bioremediation techniques, biosorption has received widespread attention due to its efficiency in the removal of toxic metals from wastewater[10]. A different group of microbes such as bacteria, fungi, and algae is involved in the biosorption and removal of mercury from the environment[11]. When compared with the other group of microbes, bacteria have the most efficient mechanism for adsorption of metals.

Bacterial communities that are exposed to mercury for long durations acquire resistance due to their property to tolerate heavy metals[12]. Bacterial strains exposed to heavy metal contamination not only acquire metal resistance, but the exposure facilitates the application of those bacteria for biosorption. Several reports are available for the biosorption of heavy metals by marine bacteria, such as *Pseudomonas aeruginosa*and*Bacillus thuringiensis*[13], *Lysinibacillus*sp, *Bacillus cereus, Kocuriarosea, Microbacteriumoxydans, Serratiamarcescens,* and *Achrobactrium*sp.[14]. Although various studies have been conducted so far regarding the use of marine bacteria for boisorption of heavy metals, only few studies have been carried out on the biosorption capability of bacteria collected from mangrove environment. Mangroves are one of the most protective and biologically richest ecosystems. They are the reservoirs of wide range of microorganism.

Exploration on microbial diversity in the mangrove sediments are important in understanding the process of bio-geochemical cycling and remediation of pollutant[15] (Roy et al. 2002). Mangrove microorganisms have proven to be an important source of nutrition, drug and antibiotic compounds[16] (UshaKiranmayi 2005). They serve as an important source of biotechnologically valuable products. The halotolerant and halophilic bacteria and other microbes present in the mangrove ecosystem have wide range of industrial applications[17] (Sabu 2003). These are prominent ecosystems which contribute to major quantities of organic load through litter fall. The rich organic matter present in the mangrove ecosystem acts as an effective binding agent for heavy metals[18]. The bacteria present in the mangrove sediments apparently have the ability to resist heavy metals. Considering the significance of mangrove associated microbes, the potential mercury-resistant bacterium was screened from the mangrove environment and the biosorbing ability experiments were conducted to characterise the bacterial strain and to evaluate the mercury bio sorption ability.

Materials and Methods

The Vellar estuary flows in the southeast coast of India and joins the Bay of Bengal at Parangipettai (Lat. 11º 29' N; Long. 79º 47' E). It is a tropical, shallow, bar-built estuary having an average depth of 2.5m. The maximum width of the estuary is about 200m. A mangrove plantation covering an area of 402.8m^2 (26.5-m by 15.2 m) was developed at the banks of the Vellar estuary by CAS in Marine Biology, Annamalai University. The existing mangrove species are *Avicennia marina*, *Rhizophora apiculata,* and *R. mucronata.*

Three replicates of surface sediment samples (0–5 cm) were collected from the mangrove patches of rhizosphere sediments. A total of 12 samples was examined at four different sites at 25-m intervals by using VanVeen grab. Five samples were collected from each sampling spotand kept in an insulated box at 4°C and transported to the laboratory. Sediment samples were transferred to Zobell Marine Broth (ZMB) supplemented with mercuric chloride ($HgCl_2$) andkept in an orbital shaker at 200 rpm for 5 days. Further, the bacterial inoculums were transferred to Zobell Marine Agar 2216 (ZMA) plates with a supplement of 2.0mgL^{-1} inorganic mercury (as $HgCl_2$) in Milli Q water and then incubated for 24 h at 37°C. Highly resistant strains were obtained by repeated streaking onto plates containing similar medium compositions (ZMA and mercury concentration) and pure strains were used for subsequent experiments [14].

The highly tolerant bacterial colonies were screened using minimal inhibitory concentration (MIC) test. MIC values of different bacteria were determined using the plate diffusion method. ZMA plates were prepared, four wells are made and each well was filled with 0.5 ml of of mercuric chloride at four different concentrations (25, 50, 100 and 200mg^{-1}). Then the plates were incubated at 37ºC for 24h to allow diffusion of mercuric chloride into the agar. Pre-incubation time facilitates the formation of a concentration gradient in the medium around the well. Plates were inoculated with different bacterial strains then incubated at 37ºC for 7 days depending on the culture conditions of each strain. After incubation, area of growth inhibition (mm) was measured as the distance from the edge of the well to the leading edge of the growing colonies. Potential mercury-tolerant bacterium was selected by measuring the highest zone of inhibition[19].

The potential bacterial strain was screened based on the highest MIC value and named as CASKS3. Morphological identification and biochemical characterization was done by following the method described in Bergey's Manual of Determinative Bacteriology[20].

The culture of selected strain CASKS3 was grown in Zobell marine broth. Total DNA was extracted following the procedure of Sambrook et al. (1989)[21]. 16S rDNA was amplified from the extracted genomic DNA using the following universal eubacterial 16S rRNA primers: forward 5' AGAGTTTGATC CTGGCTCAG 3' (*Escherichia coli* positions 8−27) and reverse 5' ACGGCTACCTTGTTACGACTT 3' (*Escherichia coli* positions 1494−1513)[22].

The amplified products (~1500 bp) were purified individually using HiYield™ Gel/PCR DNA Extraction kit (Eurofins, Bangalore). Sequences obtained were assembled, analyzed, and manually edited using MEGA 5.0 software package and compared with sequences in the NCBI database (http://www/ncbi.nlm.nih.gov/) using local alignment carried out using CLUSTALX[23].

Antibiotic susceptibility test for the selected bacterium CASKS3 was performed against eight different antibiotics viz. amikacin 10mcg, amoxicillin 10mcg, bacitracin 10mcg, cephalothin 30mcg, erythromycin 15mcg, novobiocin 30mcg, oxytetracycline 30mcg, vancomycin 30mcg, penicillin 30mcg, levofloxacin 30mcg, and cephalothin 30mcg using the method described by CLSI[24].

Overnight culture was used as an inoculum (approximately 10^{-6}/ml) for evaluating metal resistance of the potential isolate CASKS3. The experimental set-up for biosorption assessment was made as follows: 250ml flasks containing 100ml of zobell marine broth supplemented with mercuric chloride at concentrations of 200, 400, 600 and 800mg^{-1} were inoculated with 1ml of inoculum. Each of the sets was prepared in triplicate. One set without mercuric chloride was inoculated as a control. Flasks were incubated at 37°C for 48h. Optical density (OD) of the culture broth was observed at 2hr, 4hr, 8hr, 16hr, 24hr and 48hrs of incubation at 600nm using a spectrophotometer (SHIMADZU UV 1800) modified method of Khodaverdiloo and Samadi (2011)[25].

Samples from different incubation periods were collected at periodic time intervals and centrifuged at 1000 rpm for 20 min. The supernatant and pellets of each incubation period were separated further and the culture supernatants of CASKS3 were taken for residual mercury analysis. Samples were digested with nitric acid (HNO_3) and sulfuric acid (H_2SO_4). The digested samples were analyzed for the estimation of mercury using a cold vapor mercury analyzer (Model MA 5840).

Biosorption of mercury by a bacterium was characterized with the help of Fourier transformed-infrared (FT-IR) analysis (Model -Iraffinity^{-1}, Japan). Analysis was done using 0.5ml of the liquid culture, which had been treated with mercury. The sample that had not been treated with mercury was used as a control. The sample was scanned in the infrared range of 400−4000 cm^{-1}.

Results

Mercury-tolerant bacteria from the sediment samples of mangroves were isolated by Zobell marine agar in the presence of 10µM of $HgCl_2$. Forty-eight colonies exhibited tolerance up to 10µM $HgCl_2$ concentrations. Six of the most tolerant and morphologically different strains from the forty-eight colonies were taken for the MIC test.Minimum inhibitory concentration was observed at four different concentrations (25, 50, 100, and 200mg^{-1}) of mercuric chloride in all six strains. The CASKS3 strain showed the minimum zone of inhibition at higher concentrations (200mg^{-1}) (Table 1).

Therefore, this strain was taken for further analysis and the biochemical characteristics of the selected strain were observed (Table 2).The selected bacterial 0258strain CASKS3 was characterized and identified as *Bacillus* sp. using standard morphological, physiological, and biochemical tests. This strain CASKS3 was subjected to 16SrRNA gene sequence analysis for validation and the sequence was submitted to GenBank and confirmed as *Bacillus thuringiensis* and accession number (KM186604) was obtained from GenBank. A similar search was performed using the BLAST program, which indicated a close genetic relation of the strain CASKS3 with NCBI database. Phylogenetic

Fig. 1 — Resistance capacity of CASKS3 to mercury chloride (MIC TEST) which shows Minimum Inhibition of *Bacillus* to mercury in different concentration (25, 50, 100, 200 mg^{-1}).

Table. 1 — MIC Results of selected strains

S.No.	Strains	Zone of inhibition (cm in 100 mg^{-1})
1.	CASKS3	0.10
2.	CASKS7	0.40
3.	CASKS9	0.30
4.	CASKS10	0.40
5.	CASKS12	0.50
6.	CASKS14	0.40

Table. 2 — Morphological and biochemical characteristics of strain CASKS3

Tests	Response of the organism
Gram reaction	Positive
Shape	Rod
Pigments	Negative
Citrate utilisation	Positive
Indole	Positive
Methyl red	Positive
Nitrate reduction	Negative
Oxidase	Negative
Catalase	Positive
VogesProskauer	Positive
Glucose fermentation test	Positive
Mannitol fermentation test	Negative
Sucrose fermentation test	Positive
Starch Hydrolysis	Positive
Gelatin Hydrolysis	Positive

tree supported the fact that the selected strain was closely related to *B. thuringiensis* (Fig 2).

The isolated strain CASKS3 was also observed for multidrug susceptibility test with eight different antibiotics. Strain CASKS3 was found to be resistant to three antibiotics, namely amoxycillin, bacitracin, and cephalothin but susceptible to other antibiotics, i.e. amikacin, erythromycin, novobiocin, oxytetracycline, and vancomycin (Fig. 3).

The culture started to grow after a two-hour incubation period, and the specific growth rate gradually increased after 4 h in the media that contained different concentrations of mercury. The peak growth was observed at 16–24hrs, and after 24 hrs it started declining. A higher growth rate was observed in media that contained low concentrations of mercury in comparison to the media that contained high concentrations of mercury. (Fig. 4).

The results of residual mercury were analyzed in the broth culture of the *B. thuringiensis*(CASKS3) grown at four different concentrations for a period of 24 hours (fig. 5). The observed results showed removal efficiencies of 62.4% at 200 mg^{-1}, 54% at 400 mg^{-1}, 40% at 600 mg^{-1}, and 38% at 800 mg^{-1}. The mercury removal efficiency negatively correlated with the concentration of the media.

Fig. 2 — Phylogenetic tree of *Bacillus thuringiensis* (KM186604)

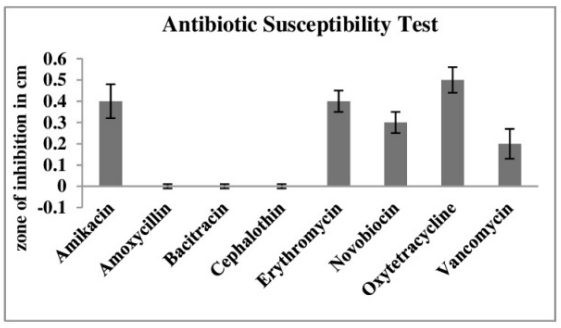

Fig. 3 — Antibiotic susceptibility of *Bacillus thuringiensis* (KM186604)

FTIR spectrum showed the specific absorption bands for carbohydrates (a broad O−H elongated band in the range of 3,200–3,700 cm^{-1} and one more band in the range of 1,000–1,200 cm^{-1} that is assigned to C−O elongation). Protein-denoting characteristic bands were also obtained (elongation of amide C=O in the range of 1,680–1,630 cm^{-1} and amide band in the range of 1,550–1,650cm^{-1}). Peak range of 1,750–650 cm^{-1} (fig. 6) denoting the variation in the concentration of carbohydrates, lipids, proteins and nucleic acids has the carboxyl group in its structure.

Discussion

Microbiological degradation is one of the main processes by which the concentration of heavy metals in wastewater is treated. The bacterial strain having the highest resistance to a particular metal can able to utilize for decreasing the toxicity level heavy metal contaminant sites[26]. In the present investigation, mercury-resistant bacterial strains were isolated from mangrove environment based on the MIC test results. Among the resistant strains, the strain CASKS3 displayed the highest tolerance to mercury. It displayed the minimum zone of inhibition at 200μg^{-ml} and it was higher than that of other bacterial strains observed in

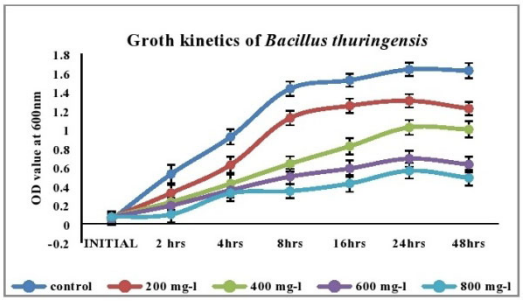

Fig. 4 — Growth patterns of *B. thuringiensis* (KM186604) grown in nutrient broth amended with 200, 400, 600, 800mg^{-1} of mercury chloride.

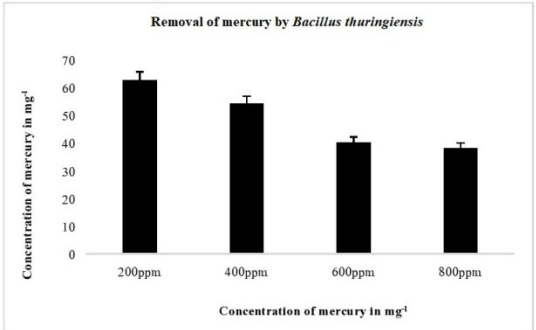

Fig. 5 — Biosorption capacity of *Bacillus thuringiensis* (KM186604)

the previous experiments[2728,29]which were isolated from industrial effluents. The mercury-resistant bacterium (CASKS3) was also resistant to some antibiotics. Earlier findings also suggested that the bacteria with a high metal resistance have been reported to have an association with antibiotic resistance[30,31]. Similarly, Mathe et al. (2012)[32] also observed the highest tolerance to metals in bacterial strains such as *Rhodococcuserythropolis, Pseudomonas corrugata*, and *P. fluorescens* collected from the sediment samples of central-east Romania have remarkable resistance against multiple antibiotics. Wright et al. (2006)[31] investigated the antibiotic resistance of bacteria from marine and freshwater sediments, agricultural soils, drinking water systems, and oral and fecal bacteria isolated from mercury-exposed primates using a culture-independent approach, and they concluded that bacteria sampled along a gradient of metal contamination were more tolerant of antibiotics and metals compared to bacteria from a reference site. The co-action of antibiotic and heavy metal-resistant genes is located on the same mobile genetic elements (MGEs) such as plasmids, transposons, and integrons[33].

*B. thuringiensis*has been used for wide range of applications and formulated into a variety of forms to be used as biological control agents. Presently, there are over 400 *Bt*-based formulations that have been registered in the market and most of them contain insecticidal proteins[34]. There have been investigations into the biosorption efficiency of *B. thuringiensis* collected from terrestrial environment. It removed 59.3% of Cr (VI) (Demir et al., 2007)[35], 16.72% of Ni (II)/g (Ozturk, 2007)[36], 83.9 % of Cd, 86.5 % of Cr, 86.1% of Cu, 79.4% of Pb, and 87.9 of Ni (Oves et al., 2013)[37]. In the present investigation, mercury resistance capability of *B. thuringiensis*isolated from the mangrove sediment was investigated at four different levels of mercury concentrations. Lowest concentration (200µg^{-ml}) was chosen based on our MIC results. The result indicated that the highest biosorption efficiency was observed at the lowest level of concentration of mercury. In terms of percentage, the removal of mercury from the solutions appeared to be more efficient at the lowest concentrations of metals (62% for 200µg^{-ml}) than at the highest concentrations (38% for 800µg^{-ml}). The low percentage of biosorption at high concentrations may be due to the insufficient free sites for metal adsorption in bacteria corroborating the observation made by various researchers[38,39,40].

The initial concentration (200 µg^{-ml}) of mercury used for the biosorption study was much higher than that in the previous studies (10µg^{-ml}) using the same species[41]. Mercury biosorption efficiency of some *Bacillus* species collected from marine and estuarine environments are presented here, 68% of mercury removal at 10µg^{-ml} by *Bacillus* sp.[42], 26% at 0.997 (mmol L^{-1}) by *Bacillus pumilus*[13], 60 % by *Bacillus cereus* and 95% by *Bacillus thuringiensis* at 50µg^{-ml}[43]. Some of the earlier result showed the greater removal efficiency than in the present study. Although, the concentration level used in other studies was lesser than that in our present experiment as shown in Table 3.

Bacterial cell walls have greater affinities for dissolved metals, and normally, metal binding take places after initial metal complexation and neutralization of the chemically active sites[11]. The functional groups responsible for heavy metal ion biosorption on *B. thuringiensis* cells are confirmed by FT-IR spectra. Several researchers have used the FTIR technique for studying the microbial compounds involved in metal adsorption. In our study, FTIR results showed various adsorption peaks in mercury-treated cells. Such bands confirm different compounds like carbohydrate,

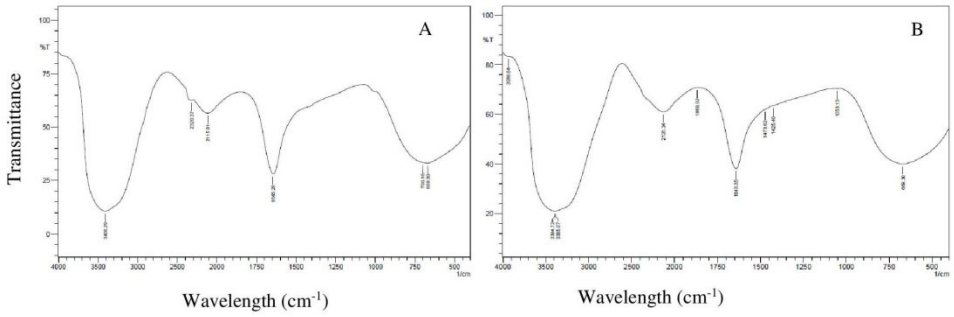

Fig. 6 (A) — FT-IR spectra of *Bacillus thuringiensis* without mercury(control),(B) FT-IR spectra of *Bacillus thuringiensis* treated with mercury solution.

Table. 3 — Comparison of biosorption efficiency of some *Bacillus* species elsewhere

S.No.	Bacterial species	Mercury removal concentrationin %	Initial mercury concentration	Source	Reference
1.	*Bacillus thuringiensis*	43%	10 μg^{-ml}	-	Hassen, 1998
2.	*Bacillus* sp.	68%	10 μg^{-ml}	Estuary	Green-Ruiz et al., 2006
3.	*Bacillus pumilus*	26%	0.997 mmol L^{-1}	Maine	Nithya et al., 2011
4.	*Bacillus thuringiensis*	95%	50μg^{-ml}	Marine	Dash and Das, 2014
5.	*Bacillus cereus*	60%	50μg^{-ml}	Marine	Dash and Das, 2014
6.	*Bacillus thuringiensis*	62%	250 μg^{-ml}	Marine	Present study

Table. 4 — FTIR stretching bond comparison for metal treated and metal untreated bacterial cells

Peaks of metal untreated bacteria	Shifted peaks of metal treated bacteria	Responsible functional group
3406.29	3385.07	-OH of alcohol group
2320.37	2131.34	C-C of alkenes
1645.28	1643.35	C=O, amide I
669.30	669.30	C-H of monosaccharides

protein, lipids, and nucleic acids (peak range from 1,750 to 650 cm^{-1}). All these have a carboxyl group in their structure Table 4). An analysis of the FT-IR spectra showed the presence of ionizable functional groups (i.e. carboxyl, amino, amide and hydroxyl) which were able to interact with protons or metal ions. Vijayaraghavan and Yun (2008)[44] stated that microbial cell walls offer specific metal-binding functional groups, such as carboxylate, hydroxyl, sulfate, phosphate, and amino groups. It should be noted that the results obtained at this stage were considered sufficient to give an idea about the presence of functional groups on the bacterial cell surfaces, which may bind with metal ions. However, it is difficult to point out the exact mechanism of mercury adsorption by bacterial biomass due to the presence of unidentified peaks in the present experiment.

Mangroves are a part of the saline coastal ecosystem and are rich in nutrients[45]. High efficiency of mercury removal by the strain *B. thuringiensis*mightbe due to its stress tolerance capacity. Bacteria isolated from the saline environment express higher resistance to heavy metals[46]and also ionic stresses[47]. Due to their prolonged stress tolerance capacity they gain the capacity to resist such stress for their own survival. The present study indicates there is a need for further investigation to screen novel strains from unexploited mangrove habitats.

Conclusion

This study validates that mercury can be effectively removed from mercurial solutions by a continuous biosorption process using *B. thuringiensis* isolated from mangrove sediments of the Vellar estuary. The strain CASKS3 is an economical and effective alternative for mercury detoxification from the contaminated environment. The results of antibiotic susceptibility test of multidrug resistance study also indicate that the strain has a narrow range of pathogenic resistance and a wide range of sensitivity against various antibiotics. Hence, this strain can be suitably used for detoxification of industrial effluents with high mercury concentrations.

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